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Evolution of Ftz protein function in insects

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The *Drosophila* gene *fushi tarazu* (*ftz*) encodes a homeodomain-containing transcriptional regulator (Ftz) required at several stages during development. *Drosophila melanogaster ftz* (*Dm-ftz*) is first expressed in seven stripes defining alternate parasegments of the embryo–a "pair-rule" segmentation function [1, 2]. It is then expressed in specific neural precursor cells in the central nervous system and finally in the developing hindgut [3]. An Orthopteran ortholog of *ftz* (*Sg-ftz,* formally *Dax*) has been isolated from the grasshopper *Schistocerca gregaria* [4]. The pattern of *Sg-ftz* expression in *Schistocerca* embryos suggests that some developmental roles of the *ftz* gene are likely to be conserved between these two species (e.g., CNS functions) while others may have diverged (e.g., segmentation functions). To test whether the function of the Ftz protein itself differs between these two species, here we compare the functions of Sg-Ftz and Dm-Ftz proteins by expressing both in *Drosophila* embryos. Sg-ftz mimics only poorly several segmentation roles of Dm-ftz (*engrailed* activation, *wingless* repression, and embryonic cuticle transformation). However, the two proteins are similarly active in the rescue of a CNS-specific *ftz* mutant. These findings argue that this *ftz* CNS function is mediated by conserved parts of the protein, while efficient pair-rule function requires sequences present specifically in the *Drosophila* protein.

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Results and discussion

A central goal of evolutionary developmental biology is to understand how animal diversity arose through the modification of developmental mechanisms. The family of *fushi-tarazu* (*ftz*) related genes of insects provides one attractive model to address this question. The *ftz* genes derive ancestrally from an Antennapedia-class gene of the Hox cluster, but they have evolved rapidly within the insects, both in terms of sequence and expression pattern.

The *ftz* gene of *Drosophila* was first identified as a "pair-rule" segmentation gene. In its absence, the embryo forms only half the normal number of segments [1, 2]. *Dm-ftz* is transiently expressed in alternate parasegments of the blastoderm stage embryo [5, 6] where it is required for the regulation of *engrailed* and other genes that define and maintain segment boundaries [7]. Later in embryogenesis, *Dm-ftz* is reexpressed in the nervous system, where it is required for the specification of certain cell fates, and finally, in the developing hindgut [3].

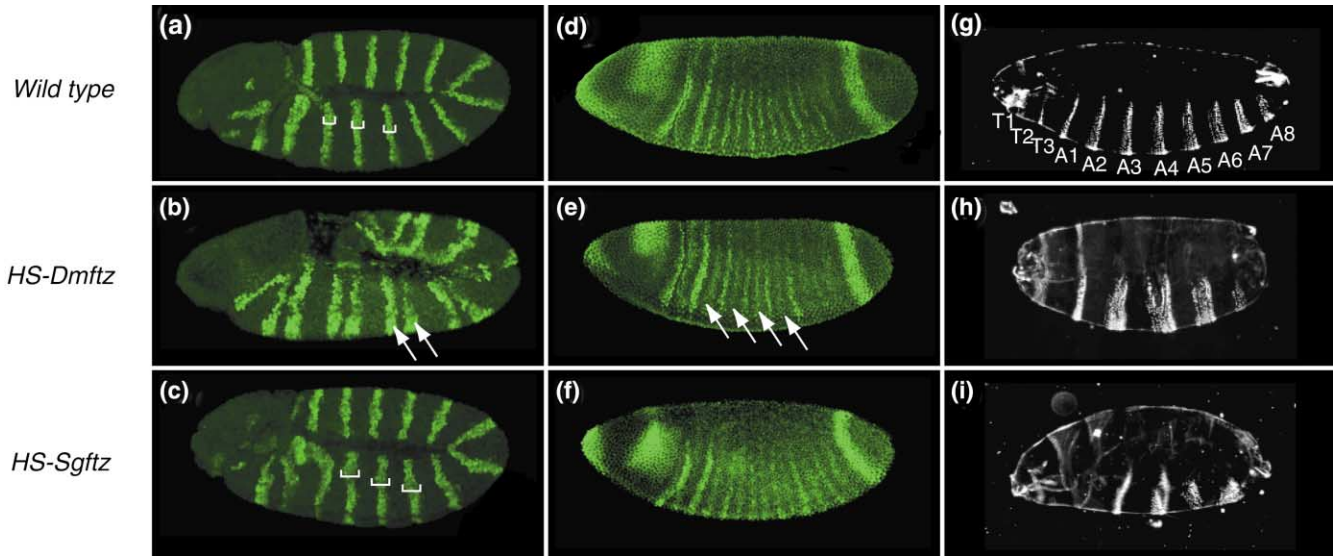
The *Dm-ftz* gene is located within the *Drosophila* Hox cluster between the genes *Sex Combs Reduced* and *Antennapedia* [2], but the sequence of *Dm-ftz* is quite divergent and does not immediately betray its ancestry. However, orthologs of *ftz* have been isolated from a number of other arthropods [4, 8–10]. Most of these retain sequence motifs that are characteristic of the Hox genes, but which have been lost from the *Dm-ftz* gene (e.g., the YPWM motif).

Pair-rule patterns of *ftz* expression similar to that seen in *Drosophila* have been observed in other holometabolous insects, though even within this group not all the developmental roles of the *ftz* genes are conserved. In the beetle *Tribolium*, *ftz* is expressed in pair-rule stripes [8], but *ftz* mutations do not prevent segment formation [11].

In *Schistocerca*, a representative of a more basal order of insects, the *ftz* ortholog is transiently expressed throughout the trunk primordium during early embryogenesis, but it never shows a spatially regulated pattern of expression analogous to that of the *Drosophila* pair-rule genes [4]. However, during the subsequent development of the nervous system, the *Drosophila* and *Schistocerca ftz* genes are expressed in strikingly similar patterns, labeling specific cells that are clearly homologous between these two species [4, 12, 13].

These observations suggest that the *ftz* family of genes has acquired new developmental roles in the lineage leading to *Drosophila*, and that these new roles may be mediated in part by changes in the structure of the Ftz protein.

Figure 1



Effects of Ftz proteins on *Drosophila* segmentation. **(a–f)** Antibody stainings and **(g–i)** embryonic cuticle preparations of *Drosophila* embryos expressing (b,e,h) Dm-ftz, (c,f,i) Sg-ftz, and (a,d,g) wild-type controls. (a–c) *engrailed* activation is shown. (a) heat-shocked wild-type embryo showing 14 evenly spaced En stripes. (b) Dm-ftz ectopic expression activates *en* expanding even-numbered stripes anteriorly generating "paired" stripes (arrows). (c) Ectopic expression of Sg-ftz does not affect the distribution of En stripes, but mildly modifies their width (brackets). (d–f) *wingless* repression is shown. (d) Wild-type control embryo stained for Wg after heat shock treatment. All the stripes show similar levels of Wg product. (e) Ectopic expression of Dm-ftz represses odd-numbered *wg* stripes (arrows). (f) Ectopic expression of Sg-ftz does not affect the wild-type *wg* pattern. (g–i) Embryonic cuticles prepared from (g) heat-shocked wild-type, (h) *HS-Dm-ftz*, and (i) *HS-Sg-ftz* embryos are shown. Ectopic expression of both Ftz proteins produced pair-rule defects, but at markedly different frequencies (36%, n = 214 and 4%, n = 232 for *HS-Dm-ftz* and *HS-Sg-ftz* embryos, respectively). Note that *Schistocerca gregaria's ftz* gene was originally termed *SgDax (Divergent Antennapedia class Homeobox gene)* [4], but now that its orthology with *ftz* is established beyond reasonable doubt [10], we propose that it be referred to as *Sg-ftz*.

It is therefore relevant to ask to what extent an Orthopteran Ftz protein can substitute for the endogenous Ftz protein of *Drosophila*. To compare the functions of these proteins, we have used transgenic *Drosophila* in which either the *Drosophila* or *Schistocerca ftz* coding sequences can be expressed ectopically at different times during development, under heat shock regulation.

Sg-Ftz functions poorly in *Drosophila* segmentation

Transgenic lines expressing the complete coding sequence of *Schistocerca* Ftz under control of a heat shock promoter (*HS-Sg-ftz*) were generated and compared with established *HS-Dm-ftz* lines [14]. Brief heat shock treatments (36.5°C, 8 min) were used to induce both gene products. After a recovery period at 25°C (30 min), embryos were fixed and stained for molecular markers or allowed to develop at 25°C until early L1 stage (20–22 hr), devitillinized, mounted in Hoyer's mountant, and examined for cuticle defects. To control for nonspecific effects caused by the temperature shock treatment, we analyzed Oregon R embryos treated in parallel to the *HS-ftz* embryos.

One of the best characterized targets of Ftz is the segment polarity gene *engrailed* (*en*) [15, 16]. Ubiquitous Ftz expression expands the boundary of even-numbered *en* stripes anteriorly, causing the stripes to appear in closely spaced pairs, rather than evenly spaced (Figure 1). Under the conditions of our experiments, ectopic expression of *Dm-ftz* leads to the pairing of engrailed stripes in 31% of treated embryos (n = 715; Figure 1b). The partial penetrance of this and other phenotypes in these experiments is due in part to the range of ages in the treated population. Eggs were collected over a period of 1 hr, but segmentation is maximally sensitive to ectopic Ftz expression for only a few minutes [14].

Overexpression of *Sg-ftz* does not induce a similar phenotype under these conditions, though it mildly affects the width of *en* stripes in a small fraction of the population (stripe width changing from 2–3 to 3–4 cells in 5% of treated embryos (n = 524; Figure 1c). Neither of these effects is seen in control embryos heat shocked in parallel (n = 613; Figure 1a).

Another target of Ftz activity is the segment polarity gene *wingless* (*wg*). Ubiquitous expression of Ftz represses *wg* transcription in odd-numbered parasegments. Kinetic ex-

periments show that this is a direct interaction [16]. We observed *Dm-ftz*-mediated repression of *wg* in 42% of the treated embryos (n = 360; Figure 1e). In contrast, *wg* expression in *Sg-ftz*-expressing embryos was indistinguishable from that seen in controls (Figure 1d,f for control and *HS-Sg-ftz* animals, respectively).

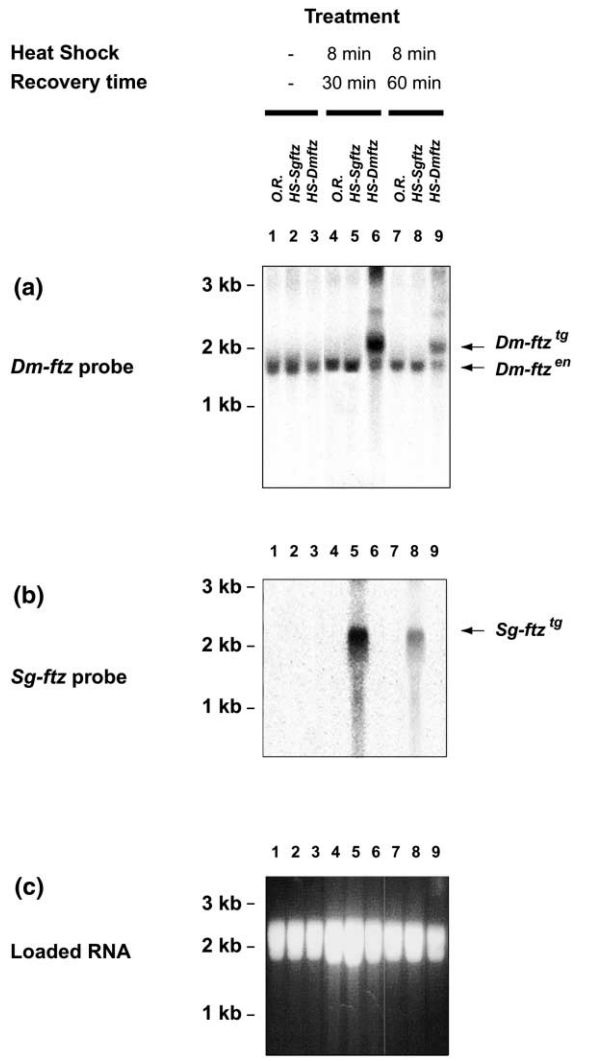
We also studied the effects of ectopic Ftz expression on the larval cuticle pattern, secreted at the end of embryogenesis. Ectopic expression of *Drosophila* Ftz protein leads to a strong "*anti-ftz*" phenotype characterized by the elimination of odd-numbered parasegments—a pair-rule phenotype [14] (Figure 1h). The *Schistocerca* protein was also able to produce pair-rule cuticular phenotypes, but only in a much smaller proportion of the embryos (4%, n = 232 for *HS-Sg-ftz* compared to 36%, n = 214 in the case of *HS-Dm-ftz*; Figure 1g versus 1i).

All of the assays above suggest that the activity of the *Sg-ftz* construct is significantly lower than that of *Dm-ftz*. One explanation for the different activity of the two constructs could be that Ftz protein levels reached after heat shock treatment were not comparable. Western blots show that Sg-ftz is clearly induced after heat shock, but neither of the available antisera for Ftz proteins [3, 4] crossreact in both *Drosophila* and *Schistocerca*, so they cannot be used to compare levels between constructs.

Northern blot experiments (Figure 2) show that the mRNA levels of both *ftz* transgenes reach a comparable value after heat shock induction, at least in so far as can be judged from blots hybridized with comparable but different probes (Figure 2a, lanes 6 versus 2b, lanes 5). Interestingly, the blots also reveal that in the conditions assayed, the endogenous *ftz* gene is not further activated by the increase of Dm-Ftz or Sg-Ftz proteins after heat shock treatment (Figure 2a, *Dm-ftz*^{en} signal). These experiments also suggest that the overall stability of both messages after induction is comparable (Figure 2a, lanes 6 and 9 versus 2b, lanes 5 and 8).

Although reassuring, this control does not directly imply similar protein levels. The most convincing evidence that expression levels do not provide the prime explanation for the different activity of the two constructs comes from the studies below, which show that in assays for a different function, the two constructs are both effective.

Figure 2



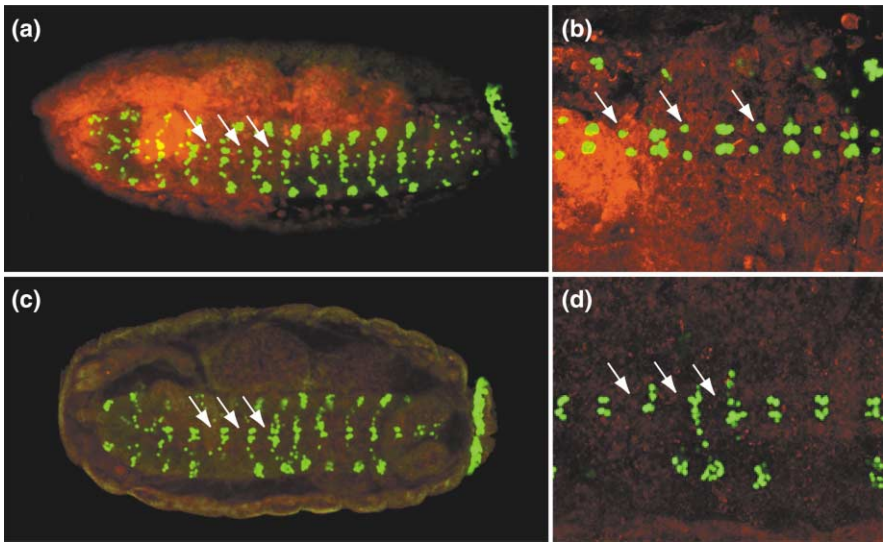
Northen blot experiment showing the expression levels of *Dm-ftz* and *Sg-ftz* messages after heat induction. Total RNA was prepared from (lanes 1, 4, and 7) Oregon Red, (lanes 2, 5, and 8) *HS-Sg-ftz*, and (lanes 3, 6, and 9) *HS-Dm-ftz* embryos (lanes 1–3) before or (lanes 4–9) after heat shock treatment. After induction, embryos were allowed to recover at 25°C for (lanes 4–6) 30 min or (lanes 7–9) 60 min before the RNA extraction was performed. RNA samples were resolved in 1.5% agarose-formaldehyde gel and transferred to a nylon membrane that was sequentially probed with **(a)** *Dm-ftz* and **(b)** *Sg-ftz* probes. **(c)** Sample loading was standardized to total RNA.

Function of Sg-Ftz in *Drosophila* CNS development

To study the activities of Dm-ftz and Sg-ftz in the *Drosophila* CNS, we used the same constructs to express these two Ftz proteins in embryos carrying a mutation that specifically disables at least one CNS function of *ftz* but has little effect on segmentation. This mutation, *ftz*$^{11.3}$ (D. Duncan et al., personal communication), replaces an arginine at position 309 in the Ftz homeodomain with a histidine (Figure 5). Homozygous *ftz*$^{11.3}$ embryos have the normal number of segments, but lack the characteristic Ftz-dependent expression of Even-skipped (Eve) protein in RP2 neurons [17, 18] (Duncan et al., personal communication; Figure 3).

We induced the ubiquitous expression of Ftz proteins in this mutant background by heat shocking embryos at 4–5 hr after egg laying, around the time that Ftz is normally

Figure 3



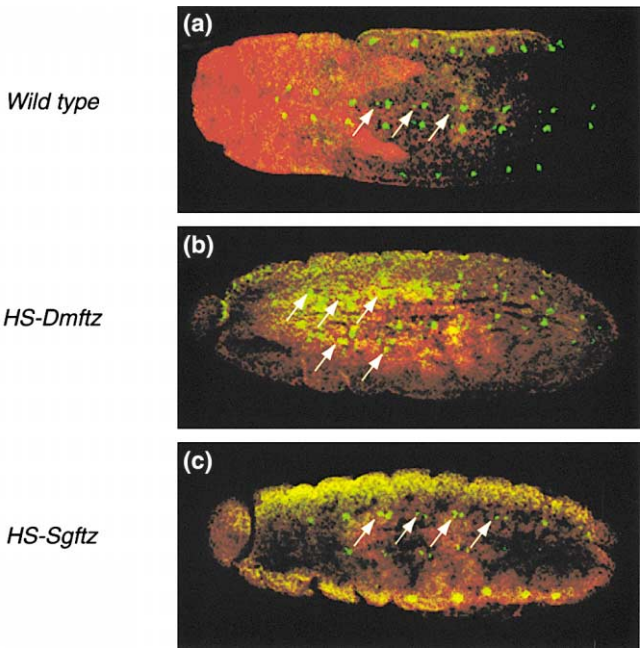
Phenotype of *ftz*$^{11.3}$ mutants. Embryos were double stained for *even-skipped* (green) and *hunchback* driven β-galactosidase proteins (red). *ftz*$^{11.3}$ homozygotes were identified by the absence of β-galactosidase signal in the head region. **(a–b)** Phenotypically wild-type embryo showing *ftz* dependent *eve* activation in RP2 (arrows) and aCC/pCC neurons. **(c–d)** Homozygous *ftz*$^{11.3}$ mutants showing loss of *eve* activation in RP2 cells (arrows). Some sporadic asymmetrically arranged *eve*$^{+}$ RP2 cells are occasionally present in a small proportion of the population (<1%). Anterior is to the left. A more detailed description of *ftz*$^{11.3}$ and other similar *ftz* mutants will be published elsewhere (D. Duncan et al, personal communication).

first expressed in the developing CNS [17]. After a recovery time of 6 hr at 25°C (Bownes stages 10–11), embryos were fixed, stained with antibody to Eve protein, and scored for the rescue of Eve-staining neurons in the normal position of RP2 cells (Figure 4). In wild-type embryos, RP2 neurons always lie anterior to the aCC/pCC neurons, which also express Eve (Figure 3).

Under these conditions, the two Ftz proteins activate *eve* in neurons at comparable, though low, frequencies. However, while Sg-Ftz activates *eve* in symmetrically located neurons at the position expected for RP2 neurons (4%, n = 260; Figure 4c), Dm-Ftz expression results in a more extensive and complex pattern of *eve* activation (5%, n = 248; Figure 4b). An analogous unrestricted expression of *eve* in CNS cells has been previously observed in certain *Polycomb* (*Pc*) group mutants [19], revealing that most CNS cells can be competent to activate *eve* expression under specific regulatory circumstances. No activation of *eve* was detected in control *ftz*$^{11.3}$ embryos heat shocked in parallel (0%, n = 470; data not shown).

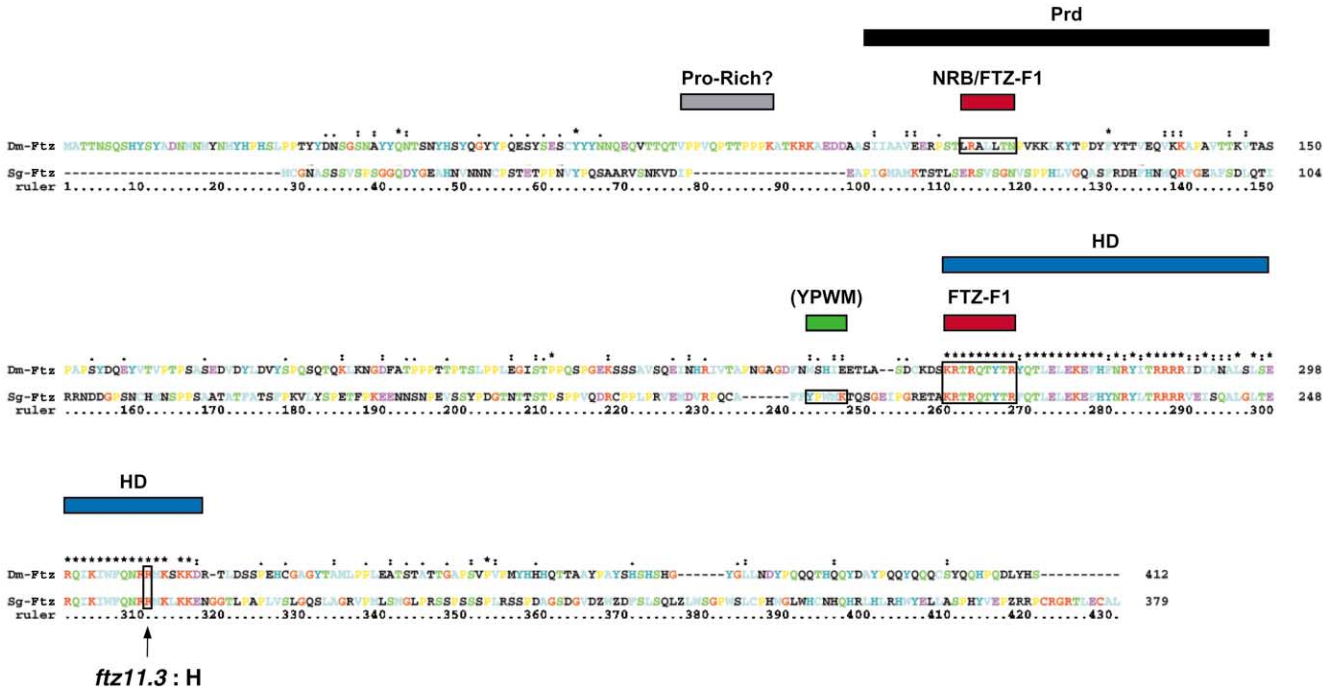
We also attempted to express Ftz proteins more specifically, only in those cells that would normally express Dm-ftz, by using a *ftz* neural promoter element to activate Ftz expression constructs via the GAL4/UAS system [20]. However, we found that even in wild-type embryos, the available *ftz*-neurogenic driver element *Nftz-Gal4* [21, 22] did not activate a *UAS-GFP/lacZ* reporter in RP2 cells labeled by Eve, while it did drive reporter expression in many other *ftz*$^{+}$ neural cells such as MP2, MP1, GP, and aCC/pCC (data not shown). Therefore, this construct cannot be used with any confidence to rescue *eve* expression by driving Ftz protein expression in RP2 cells.

Figure 4



Effects of Dm-ftz and Sg-ftz on CNS development. Embryos were double stained for Eve (green) and β-galactosidase (red) proteins. **(a)** Heterozygote *ftz*$^{+}$/*ftz*$^{11.3}$ control embryo showing *eve* expression in aCC/pCC and RP2 neurons (arrows). **(b)** Heat-shocked *HS-Dm-ftz*; *ftz*$^{11.3}$ homozygote embryos showed ectopic activation of *eve* in a complex array of CNS cells (arrows). **(c)** Heat-shocked *HS-Sg-ftz*; *ftz*$^{11.3}$ homozygote embryos showing Eve expression in symmetrically positioned CNS cells that resemble RP2 neurons (arrows). Both Ftz proteins produced these CNS effects at similar frequencies. As in Figure 2, *ftz*$^{+}$ chromosomes were marked with *hb-lacZ* to allow the detection of homozygote *ftz*$^{11.3}$ embryos. Anterior is to the left. At the posterior end of the embryos the CNS is out of the focal plane.

Figure 5



Aminoacid sequence of wild-type and mutant Ftz proteins. *Drosophila melanogaster* and *Schistocerca gregaria* Ftz proteins were aligned using *Clustal X* followed by manual amendment. Note the high level of conservation in the homeodomain region (blue box). Outside the HD, each Ftz protein contains specific protein-protein interaction modules. Dm-Ftz possess two Ftz-F1 interaction domains (red boxes). One of them is located outside the HD and is absent from the Sg-Ftz protein (NRB/FTZ-F1). On the other hand, Sg-Ftz possess an Exd/Pbx interaction domain (YPWM motif) that is absent from the *Drosophila* protein (green box). The presence of a paired-interaction region (black box) and of a putative transactivation domain of the proline-rich class (gray) in the Dm-ftz is also indicated. In the CNS-specific *ftz* mutant *ftz*$^{11.3}$, a G to A transition replaces the Arg309 by His (arrow). Slashes indicate gaps that were introduced in both sequences to maximize the alignment. Colors indicate chemical similarity of amino acids.

We sought to confirm the identity of the *HS-ftz*-induced *eve*$^{+}$ neurons using other molecular markers for RP2 cells (Zfh1 or 22C10 antibodies). However, these markers are expressed in many different neurons, so staining with these alone was not sufficient to identify the Ftz-induced cells with any certainty (data not shown). Unfortunately, the available antibodies cannot readily be used for double staining with Eve, as they are all made in the same host (mouse).

At present, therefore, the Ftz-induced *eve*-expressing neurons are only provisionally identified as induced RP2 cells. However, the fact that both *Drosophila* and *Schistocerca* proteins induce *eve*$^{+}$ cells in a comparable proportion of embryos, and that the *Schistocerca* protein specifically induces cells in the location of normal RP2 neurons, suggests that the Ftz protein of *Schistocerca* is able to replace the *Drosophila* protein for this role in CNS development. These results also suggest that the failure of the *HS-Sg-ftz* construct to activate the normal targets of Dm-ftz during *Drosophila* segmentation results from a difference in the intrinsic function of the two proteins and not simply from the inability of this construct to provide sufficient levels of protein.

Although the homeodomain (HD) of Ftz is the only part of the protein that is well conserved between *Drosophila* and *Schistocerca*, it has previously been shown to be dispensable for segmentation in *Drosophila* [23, 24]. Therefore, what sequences outside the HD might be responsible of the differential behavior of Dm-Ftz and Sg-Ftz proteins in segmentation? An alignment of these two protein sequences (Figure 5) highlights two particular modules that may confer functional specificity.

The first is a Ftz-F1 interaction domain present only in the *Drosophila* protein. Ftz-F1 is a transcription factor from the nuclear receptor family and an obligatory cofactor for Dm-ftz in segmentation functions [25, 26]. Careful deletion mapping of Dm-ftz protein has identified two interaction domains that mediate the contact with Ftz-F1 [27] (Figure 5). Interestingly, one of these domains maps outside the HD and matches the LXXLL consensus for a Nuclear Receptor Box (NRB) motif found in most

coactivators of nuclear receptors [27, 28]. This NRB/Ftz-F1 interaction domain is absent from the Sg-ftz protein (Figure 5).

The second module is a hexapeptide motif present only in the locust protein. This element is also involved in interactions with cofactors. Sg-Ftz and some other insect Ftz proteins [8] contain a short sequence generally known as the "Hexapeptide" or "YPWM motif" located N-terminal to the HD. This motif is a prefolded domain [29] present in Hox proteins and responsible for their interaction with cofactors of the TALE family such as Extradenticle/Pbx proteins [30, 31]. The *Drosophila* Ftz protein lacks the YPWM motif (Figure 5).

The presence of the Ftz-F1 interaction domain exclusively in the *Drosophila* protein and of the Hexapeptide/YPWM motif exclusively in the *Schistocerca* protein is likely to modulate the activity of these two transcriptional regulators differentially in different developmental contexts. Other, less well-defined regions that could also contribute to functional specificity are a paired interaction domain (aa 100–150) and a region of a putative transactivation domain of the proline-rich class (aa 79–90), both only present in the *Drosophila* protein (Figure 5).

In summary, our results taken together with the observations discussed above imply that one conserved role of Ftz in CNS development—*eve* activation—is mediated by the HD or other conserved sequences, while the pair-rule function of Ftz in *Drosophila* segmentation is mainly mediated by the divergent regions of the protein outside of the HD, probably through protein-protein interactions.

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